Bay or Baylike Regions of Polycyclic Aromatic Hydrocarbons Were Potent Inhibitors of Gap Junctional Intercellular Communication

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Many polycyclic aromatic hydrocarbons (PAHs) are known carcinogens, and a considerable amount of research has been devoted to predicting the tumor-initiating potential of PAHs based on chemical structure. However, there has been little research into the effects of PAHs on the epigenetic events of tumor promotion and no structural correlation has been made thereof. Gap junctional intercellular communication (GJIC) activity was used in this study as an epigenetic biomarker to determine the structure–activity relationships of twelve different PAHs. The PAHs used were naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, anthracene, 1-methylanthracene, 2-methylanthracene, 9-methylanthracene, 9,10-dimethylanthracene, phenanthrene, fluorene, 1-methylfluorene, and fluoranthene. Results showed that PAHs containing bay or baylike regions inhibited GJIC more than did the linear PAHs. The nonnaphthalene PAHs were not cytotoxic as determined by a vital dye uptake assay, but the naphthalene compounds were cytotoxic at the higher doses, indicating that the down regulation of GJIC by these naphthalenes could be a consequence of general membrane damage. Inhibition of GJIC by all the inhibitory PAHs was reversed when the cells were refreshed with PAH-free growth medium. Inhibition of GJIC occurred within 0.5–5 min and correlated with the aqueous solubility of the PAHs. The present study revealed that there are structural determinants of epigenetic toxicity as determined by GJIC activity. *Key words*: anthracenes, fluoranthene, fluorene, gap junctional intercellular communication, methylanthracenes, methylfluorene, methylnaphthalene, naphthalene, phenanthrene, polycyclic aromatic hydrocarbons. *Environ Health Perspect* 106:17–22 (1998). [Online 2 January 1998]

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Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants formed during incomplete combustion; they are also found in petroleum products and in wood-processing chemical mixtures. The PAHs are of concern because they have been shown to induce tumors in experimental animals (*1*). Occupational exposures of humans to PAH-containing products have been linked to cancer as early as 1775, when increased scrotal cancer in chimney sweeps was attributed to exposure to soot (*2*). Exposure to high levels of PAHs appears to result in an increased risk of mortality from lung, prostate, and kidney cancers as documented in exposure studies in coke oven workers and residents exposed to unvented coal or wood smoke (*3–5*). Considerable research has been devoted to predicting the carcinogenicity of PAHs based on the correlation of chemical structure and genotoxicity (*6–8*). Although the first stage of cancer (tumor initiation) often requires genotoxic/mutagenic events (*9,10*), the second stage of cancer (tumor promotion) is a consequence of a series of reversible epigenetic events (*10–13*). Therefore, epigenetic data should also play a vital role in predicting the carcinogenicity of PAHs based on chemical structure; however, such data of epigenicity is lacking in the literature.

Extracellular, intracellular, and intercellular communication play a crucial role in the epigenetic changes of gene expression (*14–18*). In particular, intercellular communication through gap junctions provides the crucial link of a cell with its neighboring cells, thus enabling individual cells to exist communally as a multicellular organism (*15–20*). Because most cancer cells do not behave in harmony with their neighbors, it is not surprising that the down regulation of gap junctional intercellular communication (GJIC) results in uncontrolled cellular growth leading to the development of tumors (*15,20*). Evidence supports the hypothesis that inhibited GJIC activity is related to carcinogenesis (*19–22*): most, if not all cancer cells, have dysfunctional GJIC; endogenous and exogenous tumor-promoting agents reversibly inhibit GJIC; oncogenes down regulate GJIC; tumor suppressor genes and antitumor promoters up regulate GJIC; and transfection of gap junction genes into GJIC-deficient and tumorigenic cells restores GJIC and normal growth regulation.

Understanding of the molecular basis of the carcinogenicity of PAHs will therefore need to include a study of epigenetic events such as those that affect GJIC. For example, newborn mice treated with three intraperitoneal doses of fluoranthene showed an increase of lung and liver tumors (*23,24*), but in other studies, there was no evidence of tumor-initiating activity

in experimental animals (*1*) or *in vivo* genotoxicity, as assessed using the mouse bone marrow micronucleus and rat liver unscheduled DNA synthesis assays (*25*). Although the tumor-promoting activity of fluoranthene has not been determined, fluoranthene was shown to inhibit GJIC in a rat liver epithelial cell line (*26*), which indicates that fluoranthene may be a tumor promoter because of its epigenetic properties.

Upham et al. (*27*) showed a structural relationship between various monomethyl isomers of anthracene and GJIC activity. The inhibitory monomethyl isomers of anthracene possess a baylike region, whereas anthracene and the monomethyl isomer with no baylike region do not inhibit GJIC. The purpose of this study was to determine if PAHs with a baylike region (formed from methyl substitution) and PAHs containing an actual bay region (formed by fused rings) are more potent inhibitors of GJIC than the more linear PAH analogues and how these compounds compare with the anthracenes. The description of bay versus baylike regions is shown in Figure 1. The aromatic hydrocarbons used in this study are naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, anthracene, 1-methylanthracene, 2-methylanthracene, 9-methylanthracene, 9,10-dimethylanthracene, phenanthrene, fluorene, 1-methylfluorene, and fluoranthene. The basic structures of these chemicals are shown in Figure 1.

These PAHs are some of the most prominent compounds of the very tumorigenic portion of cigarette smoke condensates (*28*). In particular, the most abundant PAHs in this neutral fraction were shown to be methylated anthracenes and phenanthrenes (1,494 ng/cigarette), and most of the remaining PAHs are benzo(*a*)pyrene, methylchrysene, chrysene, fluoranthene,

pyrene, methylpyrene, anthracene, and phenanthrene, ranging in concentrations of 24–360 ng/cigarette (28).

Materials and Methods

Chemicals. Anthracene, 1-methylanthracene, 2-methylanthracene, 9-methylanthracene, 9,10-dimethylanthracene, naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, fluorene, fluoranthene, 1-methylfluorene, and formaldehyde (37%) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Phenanthrene, neutral red, and lucifer yellow were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile was obtained from EM Science (Gibbstown, NJ).

Stock solutions. PAHs were dissolved in 100% acetonitrile. Concentrations of stock solutions ranged from 5 to 30 mM, depending upon the solubility of the compound in acetonitrile. The volumes of the stock solutions that were added directly to the culture medium in each plate ranged from 2 to 35 µl. Vehicle controls were added to the cells at a volume of acetonitrile equivalent to the volume of PAH stock solution used. Acetonitrile was used because it is noncytotoxic and noninhibitory up to 2% (v/v) (26). Cells were exposed to a maximum of 1.8% (v/v) acetonitrile in culture medium. Due to the low aqueous solubility of the chemicals, the maximum concentration of most of the PAHs achievable in the culture medium was 350 µM.

Cell cultures. WB-F344 rat liver epithelial cell lines were obtained from J.W. Grisham and M.S. Tsao of the University of North Carolina (Chapel Hill, NC) (29). This cell line was used because much of the *in vivo* tumor promotion assays were done in rat liver, specifically in the Fischer 344 rats. The WB-344 cell line was designed to match *in vitro* work in a liver cell line from the same strain of rat. Also the WB-344 cell line is an immortalized diploid cell line that is nontumorigenic (29), has been well characterized around the world for its expressed gap junction genes and its ability to perform GJIC via all available techniques (19), has been tested with all kinds of tumor-promoting chemicals for their ability to block GJIC (19), and has been tested for the ability of growth factors and oncogenes to modulate GJIC (19).

Cells were cultured in 2 ml D-medium (formula no. 78-5470EG; Gibco Laboratories, Grand Island, NY) and supplemented with 5% fetal bovine serum (Gibco) and 50 µg/ml gentamicin (Gibco). The cells were grown in 35-mm diameter plastic petri dishes (Corning Glass Works, Corning, NY) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Bioassays were conducted on confluent cells that were obtained after 2–3 days of growth.

Chemical treatments. In the dose–response experiments, cells were exposed to different doses of the target compound for a set period of time, and threshold values for inhibitory doses were determined. In the time–response experiments, cells were exposed for various lengths of time to the target compound at a dose that was known to cause inhibition of GJIC.

In the time recovery experiments, cells were exposed to the target compound at a dose and for a length of time that caused inhibition of GJIC. Following chemical exposure, the PAH-containing medium was discarded and cells were washed five times with phosphate-buffered saline (PBS) and recultured in fresh PAH-free medium for various incubation times.

Gap junctional intercellular communication. GJIC was determined by using the scrape loading/dye transfer (SL/DT) assay adapted from El-Fouly et al. (30). Following the chemical exposures, cells were rinsed five times with PBS, and then approximately 1 ml 0.05% lucifer yellow dye (dissolved in PBS) was added to the cells. A surgical steel blade was used to make 8–10 scrapes through the monolayer of cells. After a 3-min incubation time at room temperature, the dye was discarded and the cells were rinsed five times with PBS and then fixed with approximately 0.5 ml 4% formalin. The SL/DT assay was done immediately following chemical exposure or, with respect to the time recovery experiments, following the reincubation period.

The migration of the dye in the cells was observed using a Nikon epifluorescence phase microscope, illuminated with an Osram HBO 200 W lamp and equipped with a 35mm camera (Nikon Inc., Nikon, Japan). Photographs of the cells were taken at a magnification of 200×. Ten equally spaced measurements (1 cm apart) were made on each 200× photograph, and the distance the dye traveled was measured perpendicular to the cut. The average distance of dye migration was determined by averaging these 10 measurements and was normally 200 µm. GJIC was assessed by comparing the distance the dye traveled in the chemically treated cells to the distance the dye traveled in the vehicle controls. GJIC was reported as a fraction of the control (FOC). An FOC value of approximately 1.0 indicates normal GJIC, and FOC values less than 1.0 indicate inhibition. All experiments were done at least in triplicate, and the results were reported as mean ± standard deviation (SD) at the 95% confidence interval.

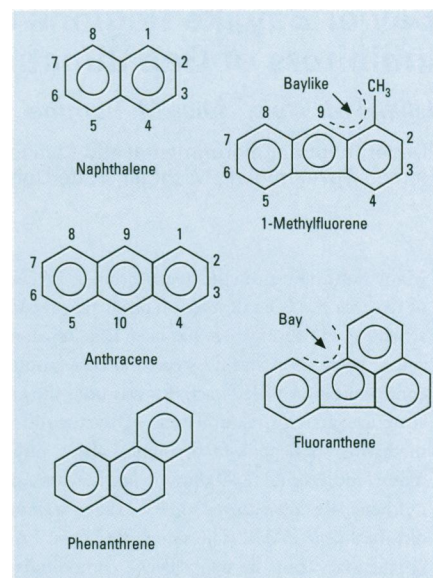*Cytotoxicity.* Cytotoxicity was determined by the neutral red dye uptake assay

Figure 1. The basic structures of polycyclic aromatic hydrocarbons used in this study.

developed by Borenfreund and Puerner (31). A solution of neutral red dye (0.033%) in D-medium was incubated at 37°C for approximately 2 hr. The neutral red solution was centrifuged at 1,300 rpm and filtered through a 0.22-µm Millipore syringe filter (Millipore Corp., New Bedford, MA). After chemical treatment, the cells were rinsed five times with PBS; the growth medium containing the neutral red dye was then added. The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 for 1.5 hr. After incubation, the cells were rinsed three times with PBS, and 2 ml of neutral red solubilizer (an aqueous solution containing 1% acetic acid and 50% ethanol) was added to lyse the cells. The solubilized dye was measured at a wavelength of 540 nm using a Beckman DU 7400 diode array detector (Beckman Instruments, Inc., Schaumburg, IL). Background absorbance at 690 nm was subtracted from the absorbance of the analyte, measured at 540 nm. Cytotoxicity was reported as FOC. The average rate of dye uptake by the control cells was 1.088 ± 0.193 OD_{540nm}/hr (mean ± SD). An FOC value of approximately 1.0 indicates that the neutral red uptake was equivalent to that of the vehicle control. An FOC value of less than 1.0 indicates less neutral red uptake as compared to the controls, and a cytotoxic response to the target compound at the concentrations tested.

Data analysis. All data were compared to and expressed as an FOC. Each value represents the mean FOC of at least three measurements from three different culture plates ± the SD at the 95% confidence interval. Curve fitting was done using
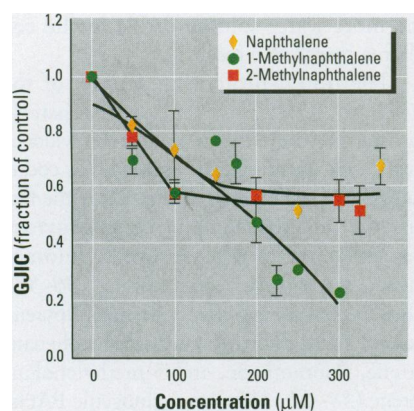
Figure 2. The effect of the naphthalene-type polycyclic aromatic hydrocarbons on gap junctional intercellular communication (GJIC). The incubation time was 4 min and the rate of dye migration in the control was 74.0 ± 5.8 μm/min (mean ± standard deviation).
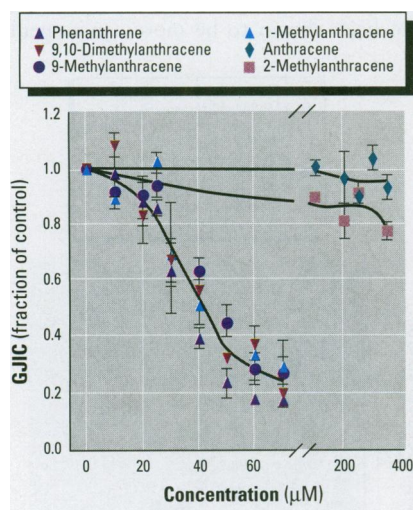

Figure 3. The effect of 3-ring polycyclic aromatic hydrocarbons on gap junctional intercellular communication (GJIC). The incubation time was 10 min and the rate of dye migration in the control was 77.5 ± 5.8 μm/min (mean ± standard deviation).
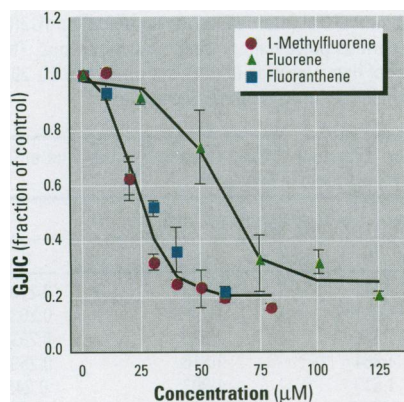

Figure 4. The effect of fluorene-type polycyclic aromatic hydrocarbons on gap junctional intercellular communication (GJIC). The incubation time was 10 min and the rate of dye migration in the control was 79.6 ± 7.0 μm/min (mean ± standard deviation).

SigmaPlot (Jandel Scientific Software, Jandel Corp., San Rafael, CA), which uses the Marquardt-Levenberg algorithm of a least squares fit.

Results

Dose response. Naphthalene and 2-methylnaphthalene partially inhibited GJIC at a dose of 350 μM (Fig. 2). 1-Methylnaphthalene completely inhibited GJIC at a concentration of 225 μM (Fig. 2). Anthracene and 2-methylanthracene did not inhibit GJIC up to a dose of 350 μM (Fig. 3). Phenanthrene, 1-methylanthracene, 9-methylanthracene, and 9,10-dimethylanthracene inhibited GJIC at 70 μM (Fig. 3). Fluoranthene and 1-methylfluorene were more inhibitory of GJIC than was fluorene (Fig. 4). Fluoranthrene and fluorene inhibited GJIC at 65 μM, whereas fluorene inhibited GJIC at 100 μM.

Cytotoxicity. The 3-ring PAHs and fluorene-type PAHs were not cytotoxic (Fig. 5) at doses that inhibited GJIC, whereas the methylnaphthalenes were cytotoxic at doses that were only slightly higher in concentration than the dose which was inhibitory to GJIC (Fig. 6). Increased incubation time of 1-methylnaphthalene at a sublethal dose resulted in an increased cytotoxic response (Fig. 7).

Time recovery. When the cells were allowed to recover in fresh medium in the absence of the target compound, GJIC was restored significantly within the first hour. GJIC was completely restored within 4 hr (Fig. 8). Recovery was similar for all of the compounds tested.

Time response. Both 1-methylnaphthalene and 2-methylnaphthalene inhibited GJIC within the first 30 sec of exposure to the target compound (Fig. 9). Inhibition of GJIC in cells treated with the 3-ring and fluorene-type PAHs occurred within 5 min (Fig. 9). A linear relationship was observed between the log octanol/water partition coefficient (log K_{ow}) and the time required for each PAH to inhibit GJIC at a value of 0.5 of the FOC. The time of inhibition at 0.5 FOC was calculated using the statistically determined values of a four parameter logistic function to describe a sigmoidal curve (Eq. 1), which was determined for each of the PAHs (Table 1), and solving for x when $f(x) = 0.5$.

$$f(x) = \frac{a}{\left[1 + e^{b(x-c)}\right]} + d \quad (1)$$

$f(x)$ is the fraction of the control, x is the incubation time of the chemical with the cells in minutes, a is the range of FOC, b is the slope coefficient, c is the inflection point of the curve, and d is the minimum FOC. Similarly, a linear regression of the relationship of log K_{ow} versus the dose at 0.5 FOC was determined for all 12 of the PAHs, generating the following equation:

$$\log K_{ow} = -0.0015(\mu M) + 4.81 \quad (2)$$

with an $r^2 < 0.081$. Clearly there was not a linear relationship between log K_{ow} and dose. Because anthracene and 2-methylanthracene did not inhibit GJIC, we used the highest dose tested; this is why we reported the r^2 value as less than 0.081.

Discussion

We have specifically compared a structural–functional relationship between the bay or baylike regions of PAHs and GJIC. The term bay region refers to the pocket formed by the sterically hindered region created by an angular benzo ring (Fig. 1). Similarly, the term baylike region is used to describe the angular pocket formed at the top of the benzene ring by a methyl group (Fig. 1). However, we should note that the bay and baylike regions discussed are not those formed by the well-known DNA reactive diolepoxides, but rather the unmetabolized
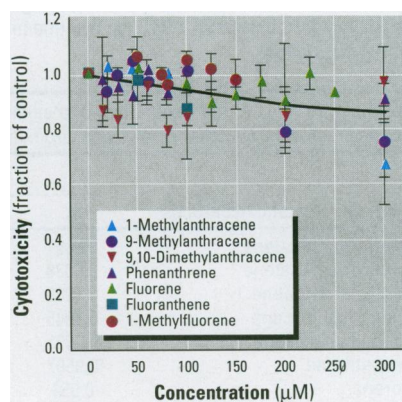

Figure 5. Cytotoxicity of anthracene-, phenanthrene-, and fluorene-type polycyclic aromatic hydrocarbons.
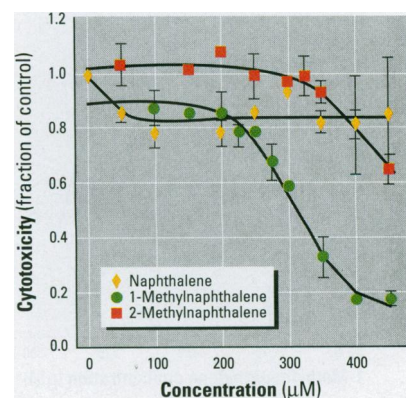

Figure 6. Cytotoxicity of naphthalene-type polycyclic aromatic hydrocarbons.

parent structure. Our focus was on the epigenetic effects of PAHs, which does not require the metabolic activation of a PAH to a chemically more reactive electrophilic compound.

The napthalene-type PAHs inhibited GJIC in a shorter time period (Fig. 2) and were cytotoxic at higher doses and incubation times (Fig. 6, Fig. 7), when compared to the other PAHs (Fig. 5). The effect of napthalene-type PAHs on GJIC could partially be due to a greater cytotoxicity, but the magnitude of inhibition was greater with 1-methylnaphthalene, which contained a baylike region. Naphthalene was shown to induce oxidative stress in rats (32); therefore, the cytotoxicity of the naphthalenes could be a consequence of oxidative stress. However, oxidative stress at noncytotoxic levels is also known to modulate GJIC (33–35).

The inhibitory effect of PAHs containing bay or baylike regions on GJIC was more pronounced with the 3-ring and fluorene-type PAHs (Fig. 3, Fig. 4). These PAHs were not very cytotoxic, even at higher doses (Fig. 5). Multiple baylike regions do not significantly increase the potency of the compound to inhibit GJIC when compared to a compound that contains a single baylike region. For example, 9-methylanthracene and 9,10-dimethylanthracene possess multiple baylike regions, while 1-methylanthracene possesses only one baylike region; yet, these compounds all had similar dose–response curves (Fig. 3). Also, there were no major differences in dose response between bay and baylike regions of fluorene-type versus the 3-ring PAHs (Fig. 3, Fig. 4). Furthermore, no significant differences were observed in the inhibition of GJIC by PAHs containing bay versus baylike regions. For example, phenanthrene and fluoranthene, which contain bay regions, exhibited similar dose–response curves as compared to 1-methylanthracene, 9-methylanthracene, and 1-methylfluorene, which contain baylike regions (Fig. 3, Fig.
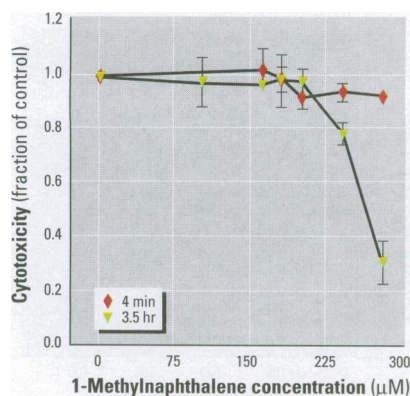
4). However, fluorene, which contains no bay region, did inhibit GJIC, albeit at a higher dose (Fig. 4). Apparently, the pentyl ring of fluorene increased the toxic effect of the 3-ring PAH. Inhibition of GJIC by these PAHs was also a reversible process (Fig. 8) which is consistent with the reversible nature of tumor promotion *in vivo* (19).

Inhibition occurred in a short period of time for all of the PAHs (Fig. 9), indicating that the gap junctions are being modified at the posttranslational level. The more water-soluble GJIC-inhibiting PAHs, which are the PAHs with lower log K_{ow} values, down regulated GJIC in a shorter time than the less water-soluble PAHs (Fig. 10). This linear relationship of log K_{ow} versus inhibition time of a PAH (Fig. 10) implies more efficient diffusion and greater bioavailability of

the more water soluble PAHs to the cell plasma membrane receptor.

The tumor-promoting activity of the PAHs we tested has not been extensively studied. Nevertheless, there is some evidence that PAHs containing bay regions are cocarcinogenic in animals, as illustrated by the fact that the carcinogenicity of benzo(*a*)pyrene was enhanced by phenanthrene, fluoranthene, and 3-methylcholanthrene (36–38). Also, the promoting effect of benzo(*a*)pyrene was enhanced by benz(*a*)anthracene, phenanthrene, fluoranthene, and 3-methylcholanthrene (38–40). These cocarcinogenic PAHs, all of which contain bay and baylike regions, are not complete carcinogens. In particular, fluoranthene and phenanthrene have been shown not to be tumor initiators (7,25, 37,41) and benz(*a*)anthracene exhibits only weak tumor initiating activity (42).

The methylated forms of PAHs have also been shown to be more carcinogenic
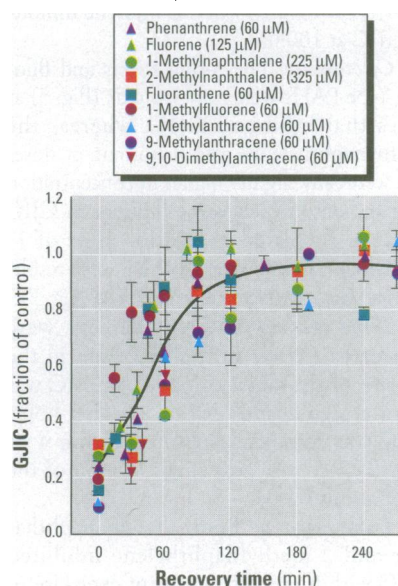
Figure 8. The recovery time after inhibition of gap junctional intercellular communication (GJIC) by polycyclic aromatic hydrocarbons (PAHs). The incubation times were 5 min for 1-methylnaphthalene and 2-methylnaphthalene, 20 min for 1-methylanthracene and 9-methylanthracene, and 10 min for all other PAHs. The rate of dye migration in the control was 95.8 ± 36.0 µm/min (mean ± standard deviation).
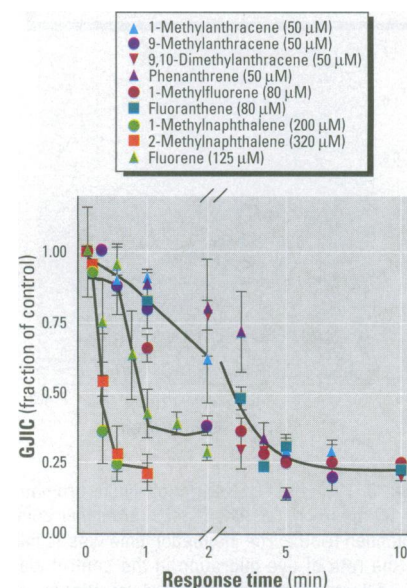
Figure 9. Time response of cells to inhibition of gap junctional intercellular communication (GJIC) by various polycyclic aromatic hydrocarbons. The rate of dye migration in the control was 89.5 ± 20.8 µm/min (mean ± standard deviation).

Figure 7. Cytotoxic dose response of 1-methylnaphthalene at two different incubation times.

Table 1. The values of the parameters of a sigmoid equation (Equation 1) used to calculate the time at 0.5 fraction of the control for each polycyclic aromatic hydrocarbon shown in Figure 10.

Polycyclic aromatic hydrocarbons	Parameters of a sigmoid equation[a]			
	a	b	c	d
1-Methylnaphthalene	0.8061	2.131	0.183	0.2105
2-Methylnaphthalene	0.9038	1.062	0.2095	0.2036
1-Methylanthracene	0.7006	1.826	1.936	0.2934
9-Methylanthracene	0.7545	1.881	1.616	0.2593
Dimethylanthracene	0.7831	1.519	1.807	0.245
Phenanthrene	0.9507	0.8556	1.961	0.1899
Fluorene	0.554	1.239	0.7618	0.3543
1-Methylfluorene	2.684	0.7432	-1.242	0.2365
Fluoranthene	0.8806	1.013	1.824	0.2314

[a]The values of the parameters were obtained by fitting a sigmoidal curve using the Marquardt-Levenberg algorithm of a least squares fit to each of the time response data in Figure 9.
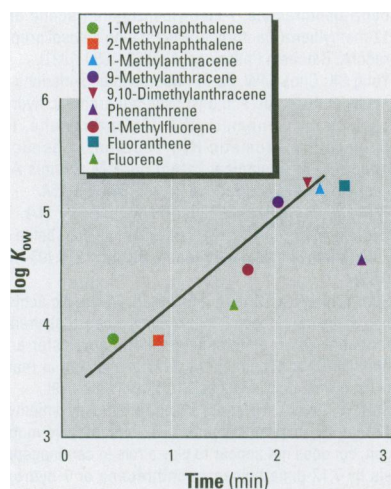
Figure 10. The linear relationship of the log K_{ow} versus the time required for a polycyclic aromatic hydrocarbon to inhibit gap junctional intercellular communication at 0.5 fraction of the control (FOC). The values of the abscissa were obtained by calculating the time in which inhibition occurred at a value 0.5 of the FOC using Equation 1 and the numerical parameters in Table 1. The K_{ow} values were obtained from Mackay et al. (*63*). The log K_{ow} = 0.60 (min) + 3.52; r^2 = 0.69.

than their parent counterparts (*7,43–50*). The increased carcinogenicity of methylated versus the unmethylated PAHs is important, considering the growing evidence that bioalkylation plays an important role in the activation pathway of converting noncarcinogenic PAHs to carcinogenic PAHs (*50–52*). Methyl substitution, which results in the formation of baylike regions, occurs *in vivo* at the mesoanthracenic positions (*43,50,51,53*). Methylated PAHs can undergo further metabolism in which microsomal enzymes hydroxylate methyl groups (*43,54,55*) and hepatic sulfotransferase enzymes catalyze the formation of sulfate esters with the hydroxymethyl groups (*55–58*).

All of the studies done to determine the carcinogenicity of methylated PAHs and their oxidized products focused on either mutagenicity or complete carcinogenicity. However, mutagenicity of strong electrophiles does not always correlate with carcinogenicity, and complete carcinogens must also induce tumor promotion through a series of epigenetic events (*59*). For example, 5-methylchrysene is more carcinogenic than the unmethylated parent compound but the higher carcinogenic potential of 5-methylchrysene and its more electrophylic metabolites could not be related to its mutagenic potential (*60*). Thus, the fraction of methylated PAHs that are not further metabolized into strong electrophiles can still exert a tumorigenic effect at the promotional/epigenetic stage of cancer. Some studies
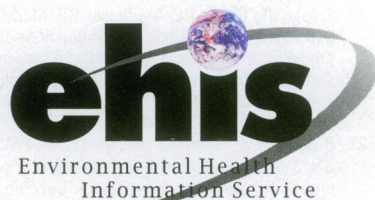
have shown PAHs to be epigenetically active, such as benzo(*a*)pyrene and 7,12-dimethylbenz(*a*)anthracene, which can increase intracellular Ca^{2+} and cell proliferation in primary human epithelial cells (*61*). Benzo(*a*)pyrene has also been shown to induce the proliferation of vascular smooth muscle cells that do not involve the mutational activation of c-*Ha*, c-*Ki*-, or N-*ras* genes (*62*). Determining structure–activity relationships relative to intracellular and intercellular communication processes involved in the promotional stages of cancer should help in predicting the carcinogenicity of the many different PAHs in our environment.

Although *in vitro* results are difficult to extrapolate to *in vivo* situations pertaining to the carcinogenic risk of a chemical, some useful information can be obtained from *in vitro* experiments. For instance, *in vitro* assays are usually better suited for studying structure–activity relationships at a more mechanistic level, which can increase our ability to predict the potency of tumor promoters based on chemical structure. Also, our *in vitro* results show a threshold level of no effect, which suggests that it should be possible to determine threshold levels when conducting *in vivo* experiments. Further, these *in vitro* studies can ultimately reduce the extent of whole-animal testing. In particular, our results show that the effects of dose, time response, and time of recovery of the 3-ring and fluorene-type PAHs on GJIC were similar (Fig. 3, Fig. 4, Fig. 8, Fig. 9). These results suggest a similar mechanism of action. Therefore, *in vivo* experiments could be minimized by randomly selecting one or two of these chemicals rather than testing all of them.

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